Ph.D. FINAL SYNOPSIS

Submitted for PhD Degree in Applied Zoology

Topic

"Molecular Taxonomy of genus *Macrobrachium* using 16s rRNA gene"

At

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Introduction

Molecular phylogenetics and systematics have shown great strides in recent years, due to the development of new and diverse methods for analysis of molecular DNA markers. These methods allow assessing the genetic variability of the biota carrying to super estimation on the global biodiversity besides the relationships among taxa (Grechko 2002). Molecular taxonomic approaches may be defined as DNA-based methods that permit an exact and rapid method of distinguishing specimens based on their interspecific variations. Freshwater prawns of the genus *Macrobrachium* (Crustacea: Decapoda: Caridea: Palemonidae) are one of the most diverse, abundant and widely distributed crustacean genera globally across the tropical and subtropical regions. Over 240 species have been assigned to this genus worldwide (Wowor *et al*. 2009). Among 60 species of *Macrobrachium* available in India (Jayachandran 2003, Jayachandran *et al*. 2010) four species were selected in the present study. Over the past 15 years, *Macrobrachium rosenbergii* (De Man, 1879) has been the subject of intensive study for aquaculture. It is the most popular freshwater shrimp for commercial culture and become the preferred prawn culture species of consumer demand attaining a length of over 300 mm. *Macrobrachium lar* (Fabricius, 1798) (monkey river prawn) attain maximum length of 181 mm. *Macrobrachium villosimanus* (Tiwari, 1949) (Dimua river prawn) attain a maximum total length male 146 mm, female 117 mm. *Macrobrachium idella* (Hilgendorf, 1898). Attain a length of 42 to 111 mm.

Many taxonomic studies have attempted to validate species identities (Holthuis 1950, 1952; Gomes-Corrêa 1977; Kensley and Walker 1982; Pereira 1997; Cai *et al*. 2004). Recently, a few efforts have focused on elucidating intrageneric relationships among species of *Macrobrachium* using molecular approaches, aiming to clarify certain questions and inconsistencies raised during the previous, morphologically based investigations. The majority of phylogenetic studies using mitochondrial genes have been conducted with Indo-Pacific species (Murphy and Austin 2002, 2003, 2004, 2005; De Bruyn *et al*. 2004; Liu *et al*. 2007). Phylogenetic relationships from DNA sequence data helps in study of taxonomy, biogeography and the evolution of life history of species. Mitochondrial DNA is commonly used in

phylogenetic studies because of several characteristics, such as its possession of both rapidly and slowly evolving regions, maternal inheritance (with some exceptions), ease of isolation, abundance, and ability to provide useful information from population to family levels (Avise *et al*. 1987; Rokas *et al*. 2003). The 16S mitochondrial DNA (mtDNA) gene region has been found to be extremely useful for studying phylogenetic relationships within a number of decapod crustacean groups (Crandall *et al*. 1999; Duffy *et al*. 2000; Schubart *et al*. 2000; Daniels *et al*. 2002; Schubart *et al*. 2002; Munasinghe *et al*. 2003). Molecular data and phylogenetic hypothesis testing have already been shown to be effective for elucidating many aspects of *Macrobrachium* systematics based on 16S rRNA (Murphy & Austin 2002, 2003; Murphy *et al*.2004;) and should allow confident insights to be made into the patterns and processes influencing the evolution of *Macrobrachium* prawns worldwide.

. The main objective of this study was to reconstruct phylogenetic relationship among *Macrobrachium* species, interspecies relationships using molecular approach aiming to characterized genetic variation of species of genus *Macrobrachium* using mitochondrial partial 16s ribosomal RNA gene. Molecular phylogenies can be used to test whether species from areas that share a common geological history are likely to have evolved in-situ or are derivatives from other regions (Benzie 1998). The present work aimed at the following:

Objectives

- To analyze the nucleotide sequence variation at 16s rRNA gene of mitochondrial DNA
- Establishment of phylogeny for *Macrobrachium rosenbergii* with other *Macrobrachium* species *Macrobrachium lar, Macrobrachium villosimanus, Macrobrachium idella.*
- To predict the evolutionary trend of different species of fresh water prawn
- Molecular taxonomy of Macrobrachium rosenbergii, *Macrobrachium idella, M. lar, M. vellisimonus*

Review of literature

The genus *Macrobrachium* can be ecologically separated into 2 groups: most species are widely distributed and require a certain saline concentration (i.e., 10 to 35 ppt) to complete their larval development, as euryhaline species; others are land-locked species, with limited distributions and complete their entire life cycle in fresh water (Holthuis 1950, Johnson 1973, Shokita 1979). As *Macrobrachium* migrated towards fresh water, the prawns gradually evolved several adaptive features. One of them is the abbreviated larval development achieved by reducing both the number of larval stages and the duration of the larval period (Shokita 1979, Jalihal et al. 1993). The abbreviated development of larvae in land-locked species was suggested to be a result of selective pressures for becoming established in freshwater environments (Shokita 1979, Magalhães and Walker 1988), and is a convergent phenomenon overriding phylogenetic relationships even above the generic level (Magalhães and Walker 1988). On the contrary, (Pereira and Garcia 1995) suggested that since primitive palaemonids, like *Troglocubanus,Palaemonetes*, and *Pseudopalaemon*, possess abbreviated development, it can possibly be considered a primitive trait, and the abbreviated development took place early in the origin of the family Palaemonidae, rather than being a recent process.

Because of the degree of conservation of morphological characteristics, much debate has surrounded the systematic relationships of many species within this group, and its taxonomy and phylogenetic inferences have until recently been exclusively based on comparisons of external morphological characters (Holthuis 1950 1952, Johnson 1973, Pereira 1997). Some species groups were proposed based on morphological similarities, mainly of the rostrum and 2nd pereiopod (Holthuis 1950, Johnson 1973). The phylogenetic significance of these groupings remains to be tested. Apart from their taxonomy, the phylogenetic affinities among *Macrobrachium* species are poorly understood. (Pereira 1997) carried out the first phylogenetic study based on morphological characters on the family Palaemonidae. In recent years, (Murphy and Austin 2002 2003 2004 2005) published a series of results for the phylogeny of *Macrobrachium* species based on the mitochondrial (mt)DNA fragment of the large subunit (16S) ribosomal (r)RNA gene marker. Their studies produced some interesting results and led to the generic clarification of some local species.

East Asia has a large landmass that resides within both the tropics and subtropics and has a long history of overland connections with tropical areas possessing rich species pools (Hamilton 1983, Guo et al. 1998). This region exhibits high species diversity of plant taxa (Guo et al. 1998, Qian and Ricklef 2000), and its freshwater fish fauna also represents one of the richest ichthyofaunas in the Sino-Indian region (Koltelat 1989, Banarescu 1990). Climatic changes and geographic heterogeneity have played major roles in the diversification and speciation of East Asia's biota, and it is a superior continental model for studying increases in regional diversity through allopatric speciation (Qian and Ricklef 2000).

Previous studies of the genus *Macrobrachium* in East Asia comprised regional species surveys or zoogeographic distributions, including in China (Yu 1936, Dai 1984, Liu et al. 1990, Cai and Dai, 1999), Taiwan (Hwang and Yu 1982 1983, Shy and Yu 1998), and Japan (Hayashi 2000a b c). In total, about 37 species of *Macrobrachium* are found in China (Li et al. 2003), Taiwan (Shy and Yu 1998), and Japan (Hayashi 2000a b c), and there is a relatively high level of endemicity. Some population studies of *M*. *nipponense* were based on allozyme variations and reproductive traits (Mashiko and Numachi 2000). (Shokita 1979) inferred the speciation and origin of land-locked species based on prawns from the Ryukyu Archipelago, southern Japan, and discussed the biogeography of the genus *Macrobrachium* with special reference to larval dispersal (Shokita 1985). However, little work has been carried out on the phylogenetic relationships among *Macrobrachium* species of East Asia.

For this study utilizing the mtDNA 16S rRNA marker, we attempted to investigate the phylogeny and evolution of land-locked species of the genus *Macrobrachium*, based on species from the Indo-West Pacific region and by using sequences available from GenBank. Then, they focused on species distributed in East Asia, including China, Taiwan, and Japan, including the Ryukyus. They used a combination of the 16S rRNA fragment gene to elucidate the phylogenetic relationships of East Asian species, to test if speciation patterns of endemic species in the region resulted from multiple lineages or from a single event, and to reveal any cryptic species that are difficult to distinguish using more-traditional techniques (Kowlton 2000, Hendrixon and Bond 2005, Ellis et al. 2006).

Technical Programme

1. Collection of samples (samples from *Macrobrachium rosenbergi* South east India, *Macrobrachium lar* from North east India, *Macrobrachium villosimanus* from North east India, *Macrobrachium idella* from South west India)
2. DNA isolation from tissue
3. Qualitative and Quantitative analysis of DNA samples by Agarose Gel Electrophoresis and Biophotometry
4. Primer Designing
5. The mitochondrial 16s rRNA gene amplified by PCR
6. Cloning and Sequencing of the gene
7. Sequence analysis by sequence analysis soft ware like Clustal W, Clustal omega, Mega V.5.0, Mega 6 analysis, Generunner, DnaSP v5 , ARLEQUIN V3 soft ware for population data analysis, PHYLIP (Phylogeny Inference packages), Network analysis, MDS PLOT (Multiple Dimension Scale)

Sample Collection:

Fresh specimens of *Macrobrachium rosenbergii* from Godavari river, 16.5333° N, 81.7333° E Palakollu, West Godavari, Andhra Pradesh, *Macrobrachium villosimanous* from Brahmaputra river, 26.4333° N, 90.3667° E Goalpara, Assam, *Macrobrachium idella* from Punnamada lake, 9.5146 N, 76.3554 E Alappuzha, Kerala*, Macrobrachium lar* from Rangat, 12.5031° N, 92.9133° E central Andaman, India, were collected and preserved in 75%-95% ethanol.

DNA extraction, PCR amplification and Sequencing

Total genomic DNA was isolated from the abdominal muscle tissue using standard phenol/chloroform method (Sambrook et al., 2001). 16s rRNA gene was amplified from total genomic DNA by a polymerase chain reaction (PCR) using the conserved primers 16SAR (5`CGCCTGTTTATCAAAAACAT 3`) and 16SBR (5`CCGGTTTGAACTCAGATCATG 3`) for 16s rRNA gene. PCR was performed in 25 µl reaction volume containing 100 ng template DNA, 10 pmol of

each specific primer, 200 µM of each dNTPs, 1.0 units of Taq DNA polymerase and 1x Taq buffer containing 1.5 mM $MgCl_2$. The amplification conditions involved an initial cycle of denaturation at 95°C for 1 min, and then 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 68°C for 30 sec, followed by a final extension at 68°C for 10 min. The size and quality of the PCR products were visualised on 1 % agarose gels. The PCR amplification products were purified with Gel extraction kit (Fermentas) following the manufacturer's protocols. For all the genes, sequencing was performed in both directions for better accuracy using ABI Big DYE terminator method (Eurofins lab, Bangalore, India).

Sequence analysis

The 16s rRNA partial gene sequences obtained for each species were manually assembled using Generunner software. Assembled sequences were end-trimmed to a homologous region to avoid sequencing errors and aligned using Clustal W (Thompson *et al.,* 1997). Only those sequences with more than 550 bp in size were used for the analysis. Sequence divergence values within and between species were calculated using Kimura two Parameter (K2P) distance model implemented in MEGA V.5.0 (Tamura *et al.,* 2011) software. The pairwise deletion option was selected to account for missing sequence information between each compared specimen. The Neighbour-joining (NJ) trees of K2P distances were created to provide a graphical representation of the divergence pattern between species and haplotypes (Saitou & Nei, 1987) with 1000 bootstrap replications. Additional elasmobranch sequences were mined from BOLD and NCBI GenBank (www.ncbi.nlm.nih.gov/) to study the allopatric divergence / species lineages, GenBank accession numbers, species names and specimen collection localities are given in Table 1. Mitochondrial 16S ribosomal RNA gene sequences were aligned using Clustal Omega [18] and sequence composition was estimated using MEGA6 [22]. Haplotype (Hd) and nucleotide (π) diversity for each population was estimated using DnaSP v5 [9]. Molecular diversity indices were determined using Arlequin V3 [3].

Results:

Mitochondrial partial 16S ribosomal gene (~480 bp) was amplified from all specimens and the resulted amplicons were purified and sequenced in both orientations. The quality of DNA sequences (~480bp) was verified by phred score (Q>30) of each individual base. All sequences were submitted to NCBI, GenBank with accession numbers of KJ544670- KJ544729 (Table 1). Out of 481 bases, the number of variable sites was for *M. rosenbergii*, *M. villosimanus*, *M.lar* and *M. idella* are 236, 34, 119 and 105, respectively (Table 2). The average nucleotide frequencies were A: 35.1; T: 29.8; C: 22.4, G: 12.6% and the sequences were found to be A+T rich (64.9%), with T_s/T_v ratio of 27.

Mitochondrial partial 16s ribosomal DNA was used for deducing the evolutionary relationship between selected *Macrobrachium* species. In total, out of 517 characters, 361 characters are variable and 331 characters are parsimony informative. This confirms that the sequences have sufficient phylogenetic signal to study evolutionary relationship. The optimum model of evolution for the dataset chosen by J Model test was the Transition model of sequence evolution (Rodriguez *et al* . 1990) plus gamma distributed rate heterogeneity with a significant proportion of invariable sites (TIM3+I+G). The parameters specified under this model were nucleotide frequencies of A = 0.31, C = 0.21, G= 0.16, T = 0.30, reversal rates of change for A ↔ C = 0.3509, A ↔ G = 5.1208, A ↔ T = 1.0000, C ↔ G = 0.3509, C ↔ T = 3.2990, gamma shape distribution $\alpha = 0.617$ and a significant proportion of invariable sites (pinv = 0.20). Across all species, no substitution saturation was detected as the level of transitional base substitutions was higher than the level of transversions over all genetic distances (Fig.1). The phylogenetic trees reconstructed by four methods i.e., Distance, Maximum parsimony, Maximum likelihood and Bayesian Inference yielded similar topologies with a slight variation in bootstrap values. The maximum parsimony, Bayesian inference method resulted in soft polytomies in tree topology and these might be due to weakness of marker or less number of species. Tree topologies resulted by NJ, MP, ML and BI analysis supported the monophyly of the genus Macrobrachium with high bootstrap values. The outgroup Exopalaemon formed as paraphyletic to Macrobrachium with significant bootstrap value. Principally all species clustered

into 5 clades as per their geographic location and evolutionary relationship (Fig.2). *M.rosenbergii, M.gangeticum, M.idella, M. villosimanus, M.lamarrei, M.malcomsonii* and *M.sankollii* (Species endemic to India; India group) formed one cluster. Species from East Asia viz., *M.nipponense, M.inflatum, M.maculatum, M.shokitai, M.asperulum, M.pinguis, M.anhuiense* formed another cluster. The phylogenetic relation among species from Indo-west pacific coast (*M.lar*, *M. latimus, M.placidium, M.gracilirostre, M.neglectum, M.idae, M. mammilodactylus* and *M.lanatum*) were not resolved properly. In Bayesian inferred phylogeny, the species (*M.carcinus, M. amricanum* and *M.olfersii*) from America formed as a basal group to other species. However, the basal position of these species needs to be further verified by using more number of markers and species.

Table 1: Accession Number of *M.rosenbergii*, *M.idella*, *M.lar*

Macrobrachium rosenbergii		
H1	5	KJ544670, KJ544675, KJ544680, KJ544685 KJ544690
H2	1	KJ544671
H3	1	KJ544672
H4	1	KJ544673
H5	1	KJ544674
H6	1	KJ544676
H7	1	KJ544677
H8	1	KJ544678
H9	1	KJ544679
H10	1	KJ544681
H11	1	KJ544682
H12	1	KJ544683
H13	1	KJ544684
H14	1	KJ544686
H15	1	KJ544687
H16	1	KJ544688
H17	1	KJ544689
H18	1	KJ544692
H19	1	KJ544693
H20	1	KJ544694
Macrobrachium idella		
H1	2	KJ544757, KJ544768

H2	1	KJ544758
H3	1	KJ544759
H4	1	KJ544760
H5	1	KJ544761
H6	1	KJ544762
H7	1	KJ544763
H8	1	KJ544764
H9	1	KJ544765
H10	1	KJ544766
H11	1	KJ544767
H12	1	69
Macrobrachium lar1		
H1	1	KJ544720
H2	1	KJ544721
H3	1	KJ544722
H4	1	KJ544723
H5	1	KJ544724
H6	1	KJ544725
H7	1	KJ544726
H8	1	KJ544727
H9	1	KJ544728
H10	1	KJ544729
Macrobrachium villiosimanus		
H1- H20	1	

Table 2: Summary of mitochondrial DNA variation among species of *Macrobrachium*

Species	Number of haplotypes	Haplotype diversity	Nucleotide diversity	Tajima's D value	Number of segregating sites	Conserved
M. rosenbergii	21	0.967±0.001	0.123±0.07	-0.204	236	246
M. villosimanus	13	0.987±0.001	0.026±0.009	0.523	34	431
M. lar	10	1.0±0.002	0.106±0.08	1.08	119	364
M. idella	20	1.0±0.002	0.07±0.06	0.486	105	376

Table 3. Specimen included in the present study

Species	Location	Accession Number
Macrobrachium gangeticum	Mumbai, India	AY730054
Macrobrachium malcolmsonii	Mumbai, India	AY730050
Macrobrachium idae	China	DQ194930
Macrobrachium neglectum	China	DQ194953
Macrobrachium sankolli	Mumbai, India	AY730052
Macrobrachium mammillodactylus	China	DQ194915
Macrobrachium carcinus	Sao Paulo, Brazil	GU929464.1
Macrobrachium gracilirostre	China	DQ194924.1
Macrobrachium lamarrei	Mumbai, India	AY730051.1
Macrobrachium placidum	China	DQ194957.1
Macrobrachium lanatum	China	DQ194911.1
Macrobrachium shokitai	China	DQ194961.1
Macrobrachium olfersii	Sao Paulo, Brazil	GU929466.1
Macrobrachium americanum	Sao Paulo, Brazil	GU929463.1
Macrobrachium asperulum	Taiwan	AB250465.1
Macrobrachium anhuiense	Taiwan	AB250426.1
Macrobrachium maculatum	China	DQ194910.1
Macrobrachium pinguis	China	DQ194958.1
Macrobrachium nipponense	Basrah, Iraq	DQ656416.1
Macrobrachium latimanus	China	DQ194937.1
Macrobrachium inflatum	China	DQ194931.1
Macrobrachium lar	India	KJ544720 -743*
Macrobrachium rosenbergii	India	KJ544670 -694*
Macrobrachium idella	India	KJ544695 -719*
Macrobrachium villosimanus	India	KJ544744 – 769*

***Sequenced in the present study**

Fig.1. Sequence saturation plots for 16S ribosomal DNA

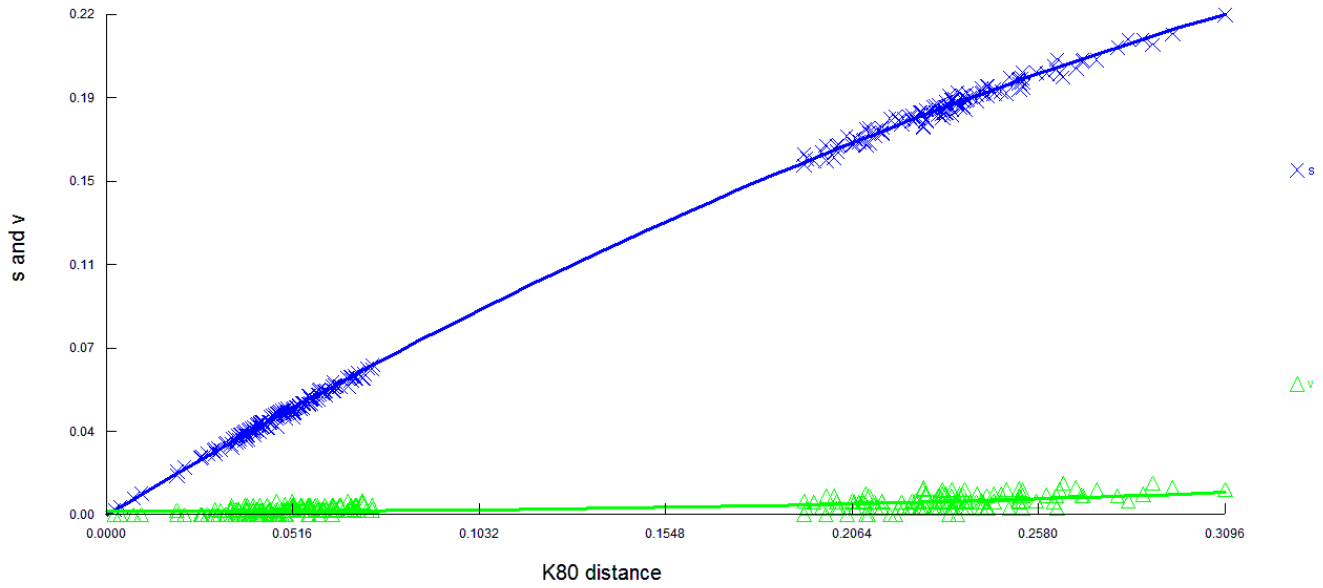
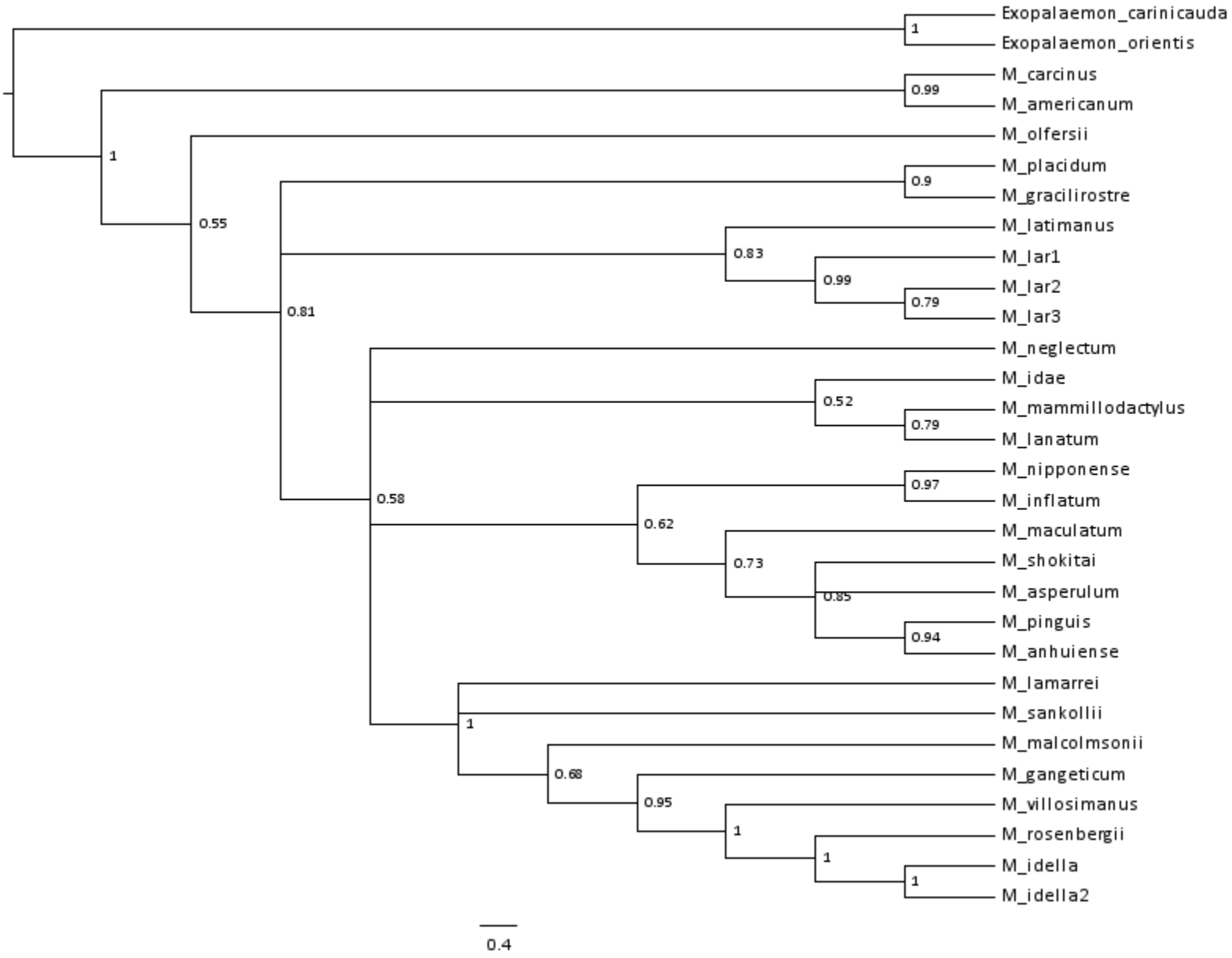


Fig.2. Phylogenetic tree (Bayesian Inference) of Macrobrachium species using mitochondrial 16s ribosomal DNA



Conclusion:

From this we can know the genetic similarity, genetic distance between species, nucleotide difference between the species of *Macrobrachium*. This study provides immense information about their phylogenetic relationship in understanding of species evolution. The phylogenetic analysis based on mitochondrial 16s rRNA gene is useful to infer relatedness between species.

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